APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1980, p. 533-538
0099-2240/80/09-0533/06$02.00/0
Vol. 40, No. 3

Limulus Amoebocyte Lysate Assay for Detection and Quantitation of Endotoxin in a Small-Volume Parenteral Product

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A *Limulus* amoebocyte lysate gel-clotting method for the determination of endotoxin in a small-volume parenteral product has been described. Sample dilution with 0.1 M potassium phosphate monobasic buffer (pH 8.0) effectively eliminated assay interference, whereas dilution with water did not. The threshold pyrogenic dose for *Escherichia coli* EC-2 and O127:B8 endotoxins was determined to be 1.0 ng of endotoxin per kg of body weight. Not more than 1.0 ng of endotoxin (the threshold pyrogenic dose) per the highest recommended human dose or the USP pyrogen test dose per kg of body weight, whichever dose is more stringent, is a logical limit for the quantity of bacterial endotoxin in small-volume parenteral products. Excellent correlation was attained when this criterion was used to compare the *Limulus* amoebocyte lysate assay with the USP pyrogen test.

Limulus amoebocyte lysate (LAL), an aqueous extract of amoebocytes from the horseshoe crab, *Limulus polyphemus*, reacts with endotoxin to form a gel or a clot (7). Under standardized conditions, this reaction detects picogram quantities of endotoxin. The clotting reaction is triggered when the LAL reagent comes in contact with the lipopolysaccharide (endotoxin) fraction of the cell wall of gram-negative bacteria. The endotoxin activates an enzyme in the LAL reagent which then reacts with a low-molecular-weight clottable protein to form a gel (17).

Testing by LAL is expeditious, specific, and convenient. The quantitative LAL test is more economical and requires a smaller volume of sample for testing than does the qualitative *United States Pharmacopeia* (USP) pyrogen test. In addition, a large number of tests can be performed by one individual in a single day. The LAL method has been approved by the Bureau of Medical Devices, U.S. Food and Drug Administration, as a suitable test to replace the USP pyrogen test for final release of medical devices (Fed. Reg. **42**:57749, 1977).

Unlike large-volume parenterals, the majority of small-volume parenteral products contain high concentrations of pharmacologically active drugs. A drug concentration of 300 to 500 mg/ml is not uncommon. The majority of these potent drugs interfere with the physiology of rabbits; thus, small-volume parenteral products tested by the USP pyrogen test must be diluted before administration. Likewise, some drugs interfere with the LAL method, necessitating dilution or other modification to eliminate interference (8, 9, 14). This paper describes development of LAL methodology for a small-volume parenteral product containing spectinomycin.

MATERIALS AND METHODS

Procedure. The experimental protocol outlined by Harrison et al. and Wachtel et al. was followed (3, 16). The LAL reagent (Pyrotell; Associates of Cape Cod, Inc., Woods Hole, Mass.; lots 52-17-202 and 52-17-212), sterile water for injection (The Upjohn Co., Kalamazoo, Mich.; lots 668FT, 050FX, and 797GX), and *Escherichia coli* O127:B8 endotoxin (Difco Laboratories, Detroit, Mich.; lot 629151) were used. The endotoxin stock solution contained 5 μg of endotoxin per ml of sterile water for injection. When the stock solution was stored at 2 to 4°C, the endotoxin was stable for at least 1 year. From this stock solution, fresh standards containing 0.01, 0.02, 0.04, 0.06, 0.08, and 0.10 ng of endotoxin per ml of sterile water for injection or buffer were prepared for use each day. The United States reference endotoxin, lot EC-2, was obtained from the Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

Sample preparation. Samples were prepared at the final concentration of 50 mg of spectinomycin per ml of 0.1 M potassium phosphate monobasic buffer. The pH of the solution was measured and adjusted when necessary with pyrogen-free HCl or NaOH to pH 6.0 to 8.0. One-tenth milliliter of solution was utilized in the gel clot LAL assay.

The 0.1 M potassium phosphate monobasic buffer was prepared by weighing approximately 13.6 g of pyrogen-free KH_2PO_4 into a pyrogen-free 1-liter volumetric flask and diluting to volume with sterile water for injection. The pH was adjusted to approximately pH 8.0 with either pyrogen-free NaOH or HCl. KH_2PO_4 was depyrogenated by adding 10% (vol/vol)

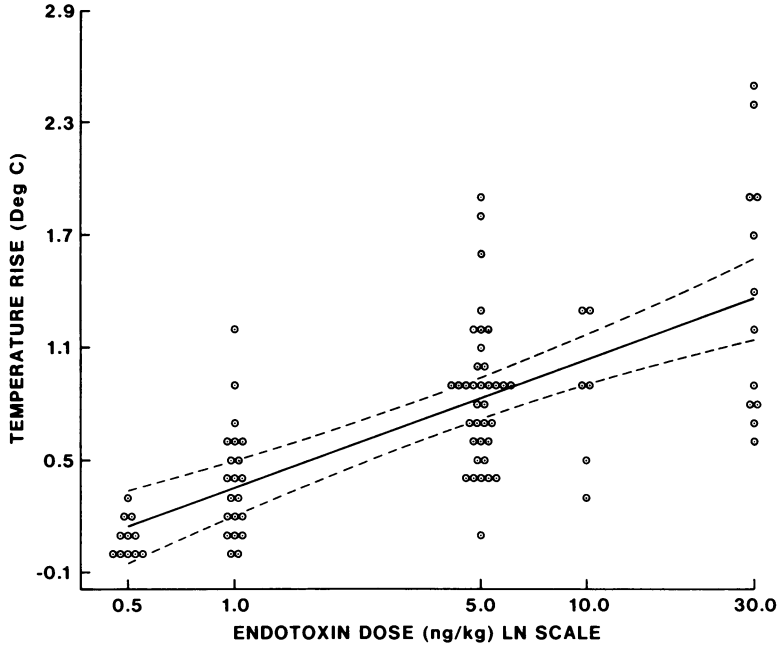

FIG. 1. *Rabbit body temperature increases recorded after administration of graded endotoxin (E. coli O127:B8) doses. Dashed lines are 95% confidence bands about the regression line.*

activated carbon (Darco G-60; ICI, United States Inc., Wilmington, Del.) to the buffer solution. The solution was autoclaved for 60 min and filtered through a 0.22-μm Millipore depyrogenated filter apparatus (Millipore Corp., Bedford, Mass.) into a pyrogen-free vessel. The pH was readjusted, when necessary, with either pyrogen-free NaOH or HCl to approximately 8.0. The buffer was utilized to dilute the endotoxin stock solution to demonstrate that the buffer does not affect the gelation endpoints. The buffer was also tested as a negative control.

Pyrogenic dose determination. To correlate the results of the LAL test with the USP pyrogen test, the threshold pyrogenic dose (TPD) was statistically determined by use of the USP pyrogen test. A group of 86 rabbits was injected with five graded doses of *E. coli* O127:B8 endotoxin: 0.5, 1.0, 5.0, 10.0, and 30.0 ng per ml of sterile sodium chloride solution per kg of rabbit body weight. To determine the TPD for the United States reference endotoxin, lot EC-2, 71 rabbits were similarly administered graded doses of EC-2 in saline. Body temperatures were recorded at hourly intervals for a minimum of 3 h after injection as outlined by the USP pyrogen test (15).

The average febrile response for each dose was a linear function of the natural logarithm of the endotoxin dose. The average pyrogenic dose (APD) was calculated as the dose at which the rabbits tested responded with average temperature increases of 0.4625°C. This value, 0.4625°C, was derived from the most stringent definition of pyrogenicity (3.7°C for eight rabbits), defined by the *United States Pharmacopeia* (15). The TPD, defined as the lower 95% confidence interval of the APD, was calculated from the linear regression analysis.

RESULTS AND DISCUSSION

TPD. Both the USP pyrogen test and the LAL assay can be used to detect endotoxins; however, only the LAL test can rapidly and precisely quantify endotoxin levels. The minimum quantity of endotoxin that is pyrogenic in rabbits, the TPD, must be determined so that the LAL assay can be correlated with the rabbit data.

Humans and rabbits are reported to respond equally on a per-kilogram-of-body weight basis to threshold pyrogenic quantities of endotoxin (2, 6). Thus, the minimum quantity of endotoxin that is pyrogenic to humans, the TPD, can be determined by the USP pyrogen test (15).

A graph of the natural logarithm of the endotoxin (*E. coli* O127:B8) dose versus the average febrile response of the 86 rabbits is presented in Fig. 1. The APD and TPD were calculated to be 1.43 ng and 1.01 ng of endotoxin per kg of body weight, respectively. Similarly, the APD and TPD were calculated for the United States reference endotoxin, lot EC-2, to be 1.37 ng and 1.05 ng of endotoxin per kg of body weight, respectively. A graph of the natural logarithm of the endotoxin (lot EC-2) dose versus the average febrile response of the 71 rabbits is presented in Fig. 2. Thus, the TPD of the two *E. coli* endotoxins was approximately 1.0 ng/kg of body weight. This value is in agreement with the finding (1.0 ng/kg of body weight) of a Health

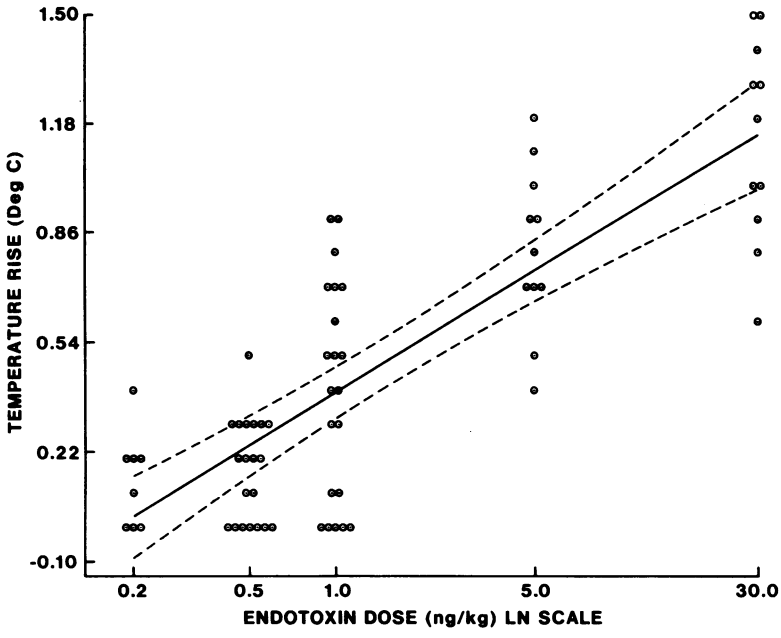

FIG. 2. *Rabbit body temperature increases recorded after administration of graded endotoxin (United States reference endotoxin, lot EC-2) doses. Dashed lines are 95% confidence bands about the regression line.*

TABLE 1. *Effect of various concentrations of spectinomycin in buffer on LAL gelation endpoints*

Spectinomycin lot	Concn of spectinomycin base (mg/ml of buffer)	Effect[a] of endotoxin concn (ng of endotoxin per ml of buffer):						
		0.10	0.08	0.06	0.04	0.02	0.01	0.00
Buffer[b]	—	+	+	+	+	−	−	−
A	50	+	+	+	+	−	−	−
B	50	+	+	+	+	−	−	−
C	50	+	+	+	+	−	−	−
D	50	+	+	+	+	−	−	−
E	60	+	+	+	+	−	−	−
E	70	+	+	+	+	−	−	−
E	80	+	+	+	+	−	−	−
E	90	−	−	−	−	−	−	−
E	100	−	−	−	−	−	−	−

[a] +, Formation of a gel that remains firm when inverted through 180°; −, no firm gel formation.
[b] The buffer utilized in this study was 0.1 M potassium phosphate monobasic buffer, pH approximately 8.0.

Industry Manufacturers Association collaborative study which used *E. coli* O55:B5 endotoxin from Difco Laboratories (4). From the statistical analysis, it was concluded that the two *E. coli* endotoxins, O127:B8 and O55:B5, are equivalent to the United States reference endotoxin, lot EC-2, and that the TPD can be defined as 1.0 ng of *E. coli* endotoxin (with reference to EC-2) per kg of body weight. Greisman and Hornick reported that the threshold pyrogenic response for both humans and rabbits is 1.0 ng/kg of body weight for an *E. coli* endotoxin (2). Hochstein et al. also established the threshold pyrogenic dose of *E. coli* endotoxin in rabbits to be 1.0 ng/kg of body weight (5).

Preparatory testing. To adequately test samples by the LAL method, the samples must not inhibit or enhance the gelation response or otherwise interfere with the LAL assay. Unlike large-volume parenterals, the majority of small-

volume parenteral products contain high concentrations of pharmacologically active drugs. A majority of these potent drugs interfere with the physiology of rabbits; thus, the products must be diluted before administration in the USP pyrogen test (12). Some drugs also interfere with the LAL test, necessitating dilution or other modification to eliminate interference (8, 9, 14).

Samples of spectinomycin were initially diluted with sterile water for injection at a final concentration of 50 mg of spectinomycin per ml, the level employed for USP pyrogen testing. The pH of these samples was adjusted with pyrogen-free NaOH to a range of between 6 and 8. The samples were then spiked with 0.05 and 0.10 ng of endotoxin per ml of sample preparation. In all cases, the formation of a gel was inhibited, even though the LAL reagent was capable of detecting less than 0.05 ng of endotoxin per ml. The interference may be due to high Na^+ content in the testing sample, since these samples required relatively large amounts of NaOH for neutralization. Sullivan and Watson (13) stated that NaCl concentrations greater than 0.154 N decrease the sensitivity of the lysate.

Dilutions of spectinomycin were next prepared in 0.1 M potassium phosphate monobasic buffer. The samples were spiked with graded doses of the *E. coli* endotoxin and tested by LAL (Table 1). Since the gelation endpoint of the endotoxin in the sample containing 80 mg of spectinomycin per ml matched the endpoint of endotoxin in the buffer, the interference shown with the LAL test in sterile water for injection was eliminated. Complete inhibition of gelation occurred for the samples containing 90 and 100 mg of spectinomycin per ml. Since the concentration of spectinomycin employed for USP pyrogen testing in rabbits is 50 mg of spectinomycin per ml, this concentration was selected for LAL testing.

Correlation of the LAL test with the USP pyrogen test. Thirty-five lots of spectinomycin were tested by LAL at 50 mg of spectinomycin per ml of 0.1 M potassium phosphate monobasic buffer. Two sets of positive controls were prepared for each lot by spiking the samples with 0.05 and 0.10 ng of the *E. coli* endotoxin standard per ml. In all cases, the lowest spiked level of endotoxin promoting a gel was the 0.05-ng/ml-spiked positive control.

USP pyrogen tests were performed on the 35 lots of spectinomycin to determine the correlation between the LAL test results and the USP pyrogen test results. In all cases, the samples were well below the threshold pyrogenic level of endotoxin; the nonpyrogenicity of the samples was confirmed by the USP pyrogen test results (Table 2).

Effect of spectinomycin on endotoxin pyrogenicity. Since no naturally pyrogenic lots were found, further testing was needed to assure that a "pyrogenic" USP test would correlate with an LAL value greater than the acceptance

TABLE 2. *Correlation of the LAL test with the pyrogen test*[a]

Lot	Endotoxin level detected by LAL (ng per 50-mg/ml dose of spectinomycin)[b]	USP pyrogen test results (maximum temp increase, °C)[c]
1	LT 0.06	0.0, 0.0, 0.1
2	LT 0.06	0.1, 0.4, 0.0
3	LT 0.06	0.2, 0.0, 0.1
4	LT 0.06	0.0, 0.2, 0.1
5	LT 0.06	0.0, 0.1, 0.2
6	LT 0.06	0.5, 0.4, 0.0
7	LT 0.06	0.0, 0.1, 0.0
8	LT 0.06	0.3, 0.0, 0.1
9	LT 0.04	0.0, 0.0, 0.2
10	LT 0.04	0.1, 0.3, 0.1
11	LT 0.04	0.0, 0.0, 0.2
12	LT 0.04	0.0, 0.2, 0.2
13	LT 0.04	0.3, 0.0, 0.4
14	LT 0.04	0.0, 0.0, 0.1
15	LT 0.04	0.0, 0.0, 0.0
16	LT 0.04	0.0, 0.1, 0.0
17	LT 0.04	0.0, 0.0, 0.1
18	LT 0.04	0.1, 0.2, 0.4
19	LT 0.04	0.0, 0.1, 0.1
20	LT 0.04	0.0, 0.0, 0.1
21	LT 0.04	0.1, 0.1, 0.1
22	LT 0.04	0.5, 0.0, 0.0
23	LT 0.06	0.0, 0.2, 0.0
24	LT 0.04	0.1, 0.0, 0.1
25	LT 0.06	0.0, 0.0, 0.5
26	LT 0.06	0.1, 0.0, 0.0
27	LT 0.04	0.0, 0.0, 0.0
28	LT 0.04	0.0, 0.0, 0.0
29	LT 0.06	0.0, 0.0, 0.2
30	LT 0.06	0.0, 0.0, 0.0
31	LT 0.06	0.0, 0.2, 0.0
32	LT 0.04	0.0, 0.0, 0.0
33	LT 0.04	0.0, 0.0, 0.1
34	LT 0.04	0.0, 0.2, 0.0
35	LT 0.04	0.0, 0.0, 0.0

[a] The positive control for all lots was 0.05 ng of endotoxin per ml of sample preparation. Samples were spiked with 0.10 and 0.05 ng of endotoxin per ml of sample preparation. The lowest endotoxin concentration that gelled is listed.

[b] The threshold pyrogenic level of endotoxin is 1.0 ng of endotoxin per kg.

[c] If no rabbit demonstrated an increase of 0.6°C or more above its respective control temperature, and if the sum of three individual maximum temperature increases did not exceed 1.4°C, then the sample was considered nonpyrogenic (15).

criterion. Determining the effects of spectinomycin on the pyrogenicity of endotoxin was also necessary to evaluate possible product synergism or inhibition. Solutions of spectinomycin were prepared at the USP and LAL testing concentration of 50 mg of spectinomycin per ml of sterile sodium chloride solution, spiked with graded doses of *E. coli* lipopolysaccharide (0.5, 1.0, and 5.0 ng of endotoxin per ml of solution), and administered to the rabbits according to the USP pyrogen test (15). Eight rabbits were tested at each dose level, and two such pyrogen assays were performed, resulting in a group of 48 rabbits tested.

The data obtained were similar and could be statistically pooled. A graph of the natural logarithm of the endotoxin dose versus the average febrile response is presented in Fig. 3. From the linear regression analysis, the APD was calculated to be 1.19 ng of endotoxin per kg of body weight. A comparison of these statistics with those of the endotoxin in sterile sodium chloride solution (APD = 1.43 ng of endotoxin per kg of body weight) (Fig. 1) indicates that spectinomycin administered in a dose of 50 mg/kg does not influence the pyrogenic activity of the endotoxin.

Endotoxin limits. The USP pyrogen test was designed to mimic the administration of the human dose (milligrams of a drug per kilogram of body weight) per injection. However, drugs are pharmacologically active compounds, and some must be diluted for testing by the USP pyrogen test to avoid rabbit sensitivity. Quite often, requirements for such dilutions are so extensive that the rabbit dose is several-fold less concentrated than the human dose.

When applied to small-volume parenteral products, an endotoxin limit should control the total, absolute quantity of endotoxin a patient receives per injected dose rather than the endotoxin per milliliter of drug solution, since the febrile response is a function of the quantity of endotoxin injected per kilogram of body weight (1, 2, 10). Administering a constant amount of endotoxin, 2 ng of *E. coli* O127:B8 endotoxin per kg of body weight, in varying volumes of saline confirmed that the febrile responses of the rabbits were similar regardless of the injection volume (Table 3). The febrile responses were a function of the total, absolute quantity of endotoxin injected per kilogram of body weight. Thus the limit of endotoxin should be based on the total quantity of endotoxin a patient receives per injection dose, e.g., the TPD, rather than the endotoxin concentration, i.e., nanograms of endotoxin per milliliter, as proposed by the Food and Drug Administration (Fed. Reg. **45**:3668, 1980).

The ultimate goal of any endotoxin or pyrogen test is protection of the patient by preventing the administration of drugs and other products

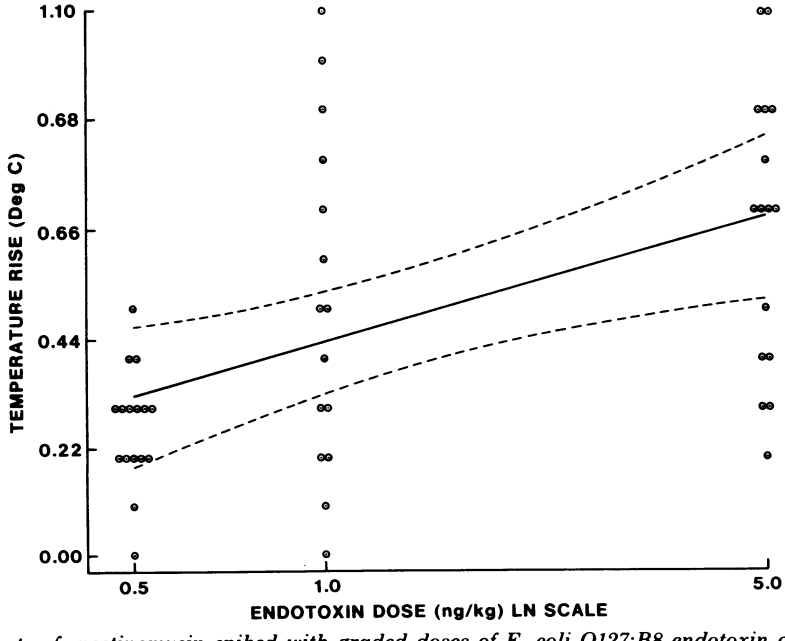

FIG. 3. *Effects of spectinomycin spiked with graded doses of E. coli O127:B8 endotoxin on rabbit body temperature increases. Dashed lines are 95% confidence bands about the regression line.*

TABLE 3. *Results of administering a constant endotoxin dose in varying volumes by the USP pyrogen test*[a]

Dose (ng of endotoxin per kg of body wt)	Dose vol (ml)	Temp increase (°C) in rabbits	Avg temp increase (°C)
2	1	0.0, 0.7, 0.7, 0.2, 1.0, 1.1, 0.6, 0.3, 0.4, 1.0	0.60
2	5	1.2, 0.7, 0.9, 0.4, 0.9, 0.4, 0.5, 0.4, 0.0, 0.2	0.56
2	10	0.9, 0.1, 0.5, 0.2, 0.6, 1.0, 0.5, 0.6, 1.1, 0.4	0.59

[a] F value: 0.03 (no statistically significant difference).

possessing high levels of bacterial endotoxins, which can produce febrile response. Thus, the limit of endotoxin in small-volume parenteral products, determined by the LAL test, should be expressed in terms of the TPD per the highest recommended human dose or per the USP rabbit dose (milligrams of a drug per kilogram of body weight), whichever is the more stringent. This proposal is often much more stringent than that being applied to medical devices (Fed. Reg. **42**:57749, 1977), which requires the LAL test for medical devices to be at least equivalent to the USP pyrogen test.

The TPD has been defined as 1.0 ng of endotoxin (United States *E. coli* reference endotoxin lot EC-2) per kg of body weight. Greisman and Hornick reported that humans and rabbits respond equally on a per-kilogram-of-body weight basis to threshold pyrogenic quantities of endotoxin, and that the TPD of *E. coli* endotoxin for both humans and rabbits is 1.0 ng/kg of body weight (2). The highest recommended intramuscular adult dose of spectinomycin is 4 g (11), or approximately 60 mg of spectinomycin per kg per injection, assuming an average human body weight of 70 kg. The limit of endotoxin, which is expressed in terms of the human dose rather than the lower USP pyrogen test dose, is thus 1.0 ng of endotoxin per 60 mg of spectinomycin.

The results of this study, as well as of those in the literature (2–4), suggest that a criterion of not more than 1.0 ng of endotoxin (referenced to lot EC-2), which is the TPD, per the highest recommended human dose or USP pyrogen test dose per kg of body weight, whichever dose is the more stringent, is a logical choice to limit the quantity of bacterial endotoxin in small-volume parenteral products.

ACKNOWLEDGMENTS

The technical assistance of K. T. Gibson, C. A. Metzer, and M. Aldrich is greatly appreciated. Statistical analyses were provided by A. R. Lewis and E. D. Williams.

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